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<u>Flow Microfluorometric Approaches to Cell Kinetics</u>

Development and application of high-speed flow systems for automated single-cell analysis of the DNA content of cell populations have provided a unique and powerful tool for use in cell-cycle studies and cell kinetic studies. Advances in instrument technology, coupled with rapid DNA staining methods requiring only 10 (Krishan 1975) or 20 (Crissman and Tobey 1974) minutes, can now be used to monitor the cycle distribution of cell populations during the course of an experiment, thus providing the investigator with an opportunity for altering a protocol at any appropriate interval. Time-sequence sampling and flow analysis of cultured cells, as well as normal and tumor cell populations, yield valuable information which reflects natural or induced changes in cell proliferation kinetics. These techniques have been used in our Laboratory to study the effects of x-irradiation on cell proliferation (Raju <u>et al</u>. 1974), the kinetics of lymphocyte population stimulation by specific antigens (Cram <u>et al</u>. 1976), and the effects of various drug agents on cell-cycle progression (Tobey and Crissman 1972, 1975a; Tobey <u>et al</u>. 1975). Investigators in many other laboratories (Hillen <u>et al</u>. 1975; Schumann <u>et al</u>. 1975; Krishan and Frei 1976) have also used flow-system techniques for analysis of cell kinetics in their own systems [see also the Proceedings of the First (Haanen <u>et al</u>. 1975) and Second (Göhde <u>et al</u>. 1976) International Symposia on Pulse Cytophotometry for additional examples].

Although the flow microfluorometric (FMF) techniques do not preclude use of standard biochemical methods of cell-cycle analysis, FMF does offer, in addition to speed and precision of analysis, several key advantages as outlined previously (Tobey and Crissman 1975b) but deserving reemphasis here. Among the advantages of the FMF procedures are the ability to (1) localize cells in S phase so as to permit distinction between early, mid, and late S phase cell populations, (2) monitor populations comprised of slowly traversing or arrested cells, (3) analyze populations devoid of cells in S phase or mitosis, (4) analyze populations containing cells unable to transport or utilize ^{3}H-thymidine, and (5) distinguish between intact and fragile (dying) cells, as will be demonstrated further in this report.

Flow-system analysis of other important cellular parameters can now be used for rapid, simultaneous analysis of cellular DNA, protein, cell volume, and light scattering of cells, with the added option of electronic sorting of particular cells fulfilling a set of predetermined biological criteria. Protein content and cell volume are important cellular properties of interest, since these parameters reflect the gross biosynthetic capacity of a cell population. Light scattering by cells yields information on cell size (Mullaney et al. 1969) and/or cellular morphology, depending on the angle used for scatter analysis (Salzman et al. 1975). Analysis of the fluorescence signals of cells stained for both DNA and protein provides a method for performing nuclear-to-cytoplasmic determinations of a cell population (Steinkamp and Crissman 1974). As an alternate approach, analysis of the fluorescence signal and small-angle light-scatter signal from DNA stained cells will also yield information concerning the comparative size relationship of nucleus and cytoplasm (Steinkamp et al. 1976).

In this report, we will present several examples that demonstrate the versatility of flow microfluorometry, with particular emphasis on use of the system for analysis of cell kinetics and/or cell growth in the _in vivo_ L1210 ascites and Lewis lung carcinoma model tumor systems as well as the _in vitro_ CHO cell culture system. Since we have previously discussed the aspects of cell dispersal, fixation, and staining (Crissman _et al_. 1975), these protocols will not be discussed in detail. Descriptions of the design and operational features of the single-parameter (Van Dilla _et al_. 1964; Holm and Cram 1973) and the multiparameter analysis and cell sorting systems (Steinkamp _et al_. 1973; Mullaney _et al_. 1974) have been discussed elsewhere.

ANALYSIS OF THE PROLIFERATION KINETICS AND PROTEIN CONTENT OF L1210 ASCITES CELLS

The L1210 leukemia was chosen as the standard model tumor reference system for screening and evaluating chemotherapeutic drugs at the 1974 World Conference on Drug Screening held in Geneva, Switzerland (Scheparts 1975). The tumor originated in 1948 in the spleen and lymph node of mice whose skin had been painted with methylcholanthrene (Geran _et al_. 1972). The tumor is propagated _in vivo_ in DBA/2 mice; however, the BDF_1 mouse (C57Bl/6 x DBS/2) is commonly used as the host for drug testing. In the present study, the L1210 ascites were grown in DBA/2 mice following an initial intraperitoneal inoculation of 10^5 cells. Under these conditions, hemorrhaging was noticeable in the peritoneal cavity on about day six or seven and was extensive at death (days eight to ten).

Mice were sacrificed at daily intervals, beginning on day two after tumor cell inoculation. Cells were harvested by aspiration, washed once in saline GM (balanced salt solution lacking calcium and magnesium) containing 0.5 mM EDTA, and fixed in 70% ethanol for $MgCl_2$ (Crissman and Tobey 1974) or for staining of both DNA and protein, respectively, using propidium iodide (Calbiochem, 0.1 mg/ml in phosphate-buffered saline) and fluorescein isothiocyanate (J. T. Baker Co., 0.05 mg/ml in 0.5 M sodium bicarbonate adjusted to pH 8.0), as previously described (Crissman and Steinkamp 1973).

Typical DNA distribution patterns obtained for mithramycin-stained populations of L1210 cells obtained on days two through seven are shown in Figure 1. Comparison of these DNA distributions to Figure 1 those of normal (diploid) spleen cells (not shown) indicated that the G_1 cells of L1210 have a 2C DNA content. The percentage of cells in G_1, S, and G_2 + M, presented graphically in Figure 2, was determined Figure 2 by computer-fit analysis of the DNA distributions using the Dean and Jett program (1974). These data and data obtained from similar experiments in our Laboratory vividly reveal changes in the proliferation kinetic patterns which are concomitant with increased cell density or tumor age.

There is an initial lag in tumor growth as indicated by the low percentage of cells in S phase on day two; however, a rapid increase in cell proliferation is apparent by days four and five, followed by a decrease in proliferating cells on day six. This dramatic decrease in cell progression capacity may result from release of cytotoxic substances during the extensive hemorrhaging which begins on day six.

The percentage of cells in S phase remains unchanged on day seven; however, there is a sizable increase in the G_2 + M fraction. It is of interest to note that the percentages of cells in G_1, S, and G_2 + M at the peak of growth on day five are quite comparable to values obtained previously in our Laboratory for exponentially growing L1210 cells <u>in vitro</u> (i.e., percentages in G_1, S, and G_2 + M, respectively, of 29.2, 65.5, and 8.3).

Examination of the DNA distribution patterns (Figure 1) reveals some fine structure worth noting. On day four, for example, there appears to be a slight but reproducible accumulation of cells in early S phase, possibly suggesting that cells are not traversing as rapidly through that portion of the DNA replicative phase (i.e., reflecting differences in the rate of DNA synthesis during that stage of tumor development). Additional studies, including cell enumeration and ^{3}H-thymidine incorporation, obviously are necessary to provide a more detailed characterization of the L1210 ascites system. Investigations involving the continuous labeling of cells with ^{3}H-thymidine, followed by sorting of cells from various phases of the cell cycle for standard autoradiography analysis, would yield information concerning non-traversing cells. The number of arrested or slowly traversing cells could be assessed, since these cells would show little or no ^{3}H-thymidine incorporation. This technique has been used previously for population analysis of arrested human diploid fibroblasts (Dell'Orco <u>et al</u>. 1975).

Simultaneous analysis of DNA and protein provides useful information relating to the biosynthetic capacity of cells at various phases of the cell cycle (Crissman and Steinkamp 1973). Since a gross imbalance in the DNA/protein ratio will eventually lead to cell death, analysis of the quantitative relationship of these parameters may be useful also for elucidating the occurrence of ensuing phase-specific cell death. Figure 3 presents single-parameter and dual-parameter DNA/protein profiles for L1210 ascites cells. The protein content distribution is similar to that obtained for cultured L1210 cells (not shown). Figure 3

We are currently using DNA/protein staining and simultaneous analysis techniques to study cell growth as well as proliferation capacity. Dual-parameter sorting techniques which permit cell sorting based on both DNA and protein content could be incorporated into the ^{3}H-thymidine and autoradiographic studies suggested above. This would provide an extremely sensitive analytical system for studying cell kinetics in tumor systems.

ANALYSIS OF DNA AND PROTEIN OF NORMAL CELLS AND TUMOR CELLS IN THE LEWIS LUNG CARCINOMA

The Lewis lung carcinoma is a solid tumor system which has also been used for drug screening. Tumors are propagated as subcutaneous (sc) implants in C57Bl/6 mice, and tests are generally performed with the BDF_1 mouse serving as host animal (Geran et al. 1972). Primary (sc) tumors grow rapidly and will eventually metastasize to the lung and other organs. This presents an interesting system for studying the growth kinetics of the tumor under differing in vivo environmental conditions. Simpson-Herren et al. (1974) have recently presented results of autoradiographic studies which demonstrated differences in the mode of proliferation of the primary tumor and lung metastases of the Lewis lung carcinoma.

DNA and protein distributions of primary tumor and lung metastases of the Lewis lung carcinoma from C57Bl/6 mice are shown in Figures 4 and 5, respectively. In preparation for these analyses, primary tumors or lung tumors were forced through 500-μm Teflon mesh into cold saline GM containing 0.5 mM EDTA. The tumor material was pipetted 20 times in and out of a 5-ml pipette and then filtered first through a 120-μm filter and then a 62-μm nylon filter. Cells were pelleted by centrifugation, washed once in saline GM plus EDTA, fixed in 70% ethanol, and stained for both DNA and protein as described for L1210 cells. Figure 4 Figure 5

Based on results obtained in both cell sorting experiments and DNA content analysis of diploid spleen cells, the peak designated 2C in the DNA distributions shown in Figures 4 and 5 is normal cells, and the second and third peaks represent, respectively, the G_1 (4C) and G_2 + M (8C) populations of the tumor cells. The percentages of cells in G_1, S, and G_2 + M were 38.7, 54.9, and 6.4, respectively, for the primary tumor and 58.8, 32.0, and 9.2, respectively, for the metastatic population. The fraction of cells in S phase for the lung metastases is in good agreement with the pulse labeling index (36%) obtained by Simpson-Herren et al. (1974) in spontaneous metastatic lung tumors 17 days after implantation of the primary (sc) tumors. That investigation yielded a much lower labeling index (26%) for primary tumors than the 54% S phase fraction obtained in our studies. However, several significant differences in experimental design of the two studies make it difficult to compare the results directly. The protein distributions of normal lung cells (dotted lines) and tumor cells (solid lines) in Figures 4 and 5 were obtained by gated analysis of the DNA distributions in regions labeled (1) and (2), respectively. Tumor cells have a greater protein mass than most normal cells.

Normal cells are present in most solid tumors where they function in various aspects of nutrition (i.e., provide adequate blood supply) or where they aid in providing a framework system for tumor architecture. In spite of the fact that the number and types of normal cells must change during the various stages of tumor development, little attempt has been made to exploit this phenomenon for characterizing the developmental stages of tumor growth and proliferation.

In the context of drug evaluation, the effects of various chemical agents on normal cells are often as important as the effects on the tumor cell population. In either instance, use of the techniques demonstrated here should be extremely useful. Sorting of cells based on DNA content measurements can provide a concentrated population composed of large numbers of normal cells for microscopic analysis. Protein content analyses of normal cells and tumor populations would be useful for determining the physiological condition of cells during drug testing experiments.

DETECTION OF FRAGILE (DYING) AND INTACT CELLS

Dead or dying cells are present in varying proportions in most tumor systems and are also found in great abundance in cell systems that have been treated with chemotherapeutic agents. Information concerning the relative proportion of dying to living cells, as well as the cell cycle phase in which cell death occurs, would be extremely useful for studying tumor cell kinetics or for elucidating the phase-specific cytotoxic effect of drugs.

We have recently devised and utilized a method for detecting dying (fragile) cells in specific phases of the cell cycle (Tobey and Crissman 1975b). The technique employs both the acriflavine-Feulgen DNA staining procedure (Tobey et al. 1972) which destroys fragile or dying cells and the mild, one-step mithramycin staining method which seems to preserve the fragile cell sufficiently for flow analysis. Figure 6 shows the DNA content distributions of Chinese hamster (line CHO) cells treated for two hours with BCNU (A), CCNU (B), or Me-CCNU (C), then re-suspended in drug-free medium and harvested four days post-drug treatment (for complete details of the study, see Tobey and Crissman 1975b). Duplicate samples of each drug-treated population were stained using the acriflavine-Feulgen procedure (open circles) or the mithramycin technique (solid circles) and analyzed. Comparison of the DNA profiles shown in Figure 6 reveals a lower proportion of acriflavine-stained cells, particularly in the 4C and 8C regions of the DNA spectra. This observation suggests that the long-term cytotoxic effect of these nitrosourea compounds is exerted primarily on the 4C and 8C cell populations. This does not preclude the possibility that cell damage was caused initially in some other phase of the cell cycle. Cells able to divide in the presence of the drug or non-traversing G_1 (2C) cells were the least affected by these compounds.

Figure 6

SUMMARY

We have presented in this report data obtained by FMF analysis of *in vivo* model tumor systems and the *in vitro* CHO cell culture system in an effort to demonstrate a few of the biological applications of flow systems, particularly for use in cell kinetics and cell growth studies. Flow systems are no longer viewed as laboratory novelty items or "electronic gadgets," as they were referred to a decade or so ago. Experiments performed in laboratories throughout the world have provided credibility and respectability to this unique methodology.

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ABBREVIATIONS

BCNU = 1,3-bis(2-chloroethyl)-1-nitrosourea

CCNU = 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea

DNA = deoxyribonucleic acid

EDTA = disodium ethylenediamine tetraacetic acid

EtOH = ethanol

Me-CCNU = 1-trans-(2-chloroethyl)-3-(4-methylchlorohexyl)-1-nitrosourea

saline GM = phosphate-buffered balanced salt solution containing 1.1 g/l glucose but lacking calcium and magnesium

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Figure 1. DNA content distributions of mithramycin-stained L1210 ascites cells grown in DBA/2 mice and harvested on days two through seven.

Figure 2. Progression of L1210 ascites cells through the G_1, S, and G_2 + M phases of the cell cycle on days two through eight.

Figure 3. Single-parameter and dual-parameter analysis of DNA and protein in L1210 ascites cells.

Figure 4. DNA, protein, and gated protein distributions of normal lung cells (-----) and tumor cells (———) of primary Lewis lung carcinoma cell populations.

Figure 5. DNA, protein, and gated protein distributions of normal cells (-----) and tumor cells (———) in lung metastases of the Lewis lung carcinoma.

Figure 6. DNA distributions of CHO cells treated with BCNU (A), CCNU (B), or Me-CCNU (C) and stained either by the acriflavine-Feulgen procedure (open circles) or the mithramycin technique (closed circles).